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Polyamine regulating protein antizyme binds to ATP citrate lyase to accelerate acetyl-CoA production in cancer cells

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ABSTRACT

Antizyme (AZ) regulates cellular polyamines (i.e., putrescine, spermidine, and spermine) through binding to ornithine decarboxylase and subsequent ubiquitin-independent degradation of the enzyme protein by the 26S proteasome. Screening for AZ-binding proteins using a yeast two-hybrid system identified ATP citrate lyase (ACLY), a cytosolic enzyme which catalyzes the production of acetyl-CoA that is used for lipid anabolism or acetylation of cellular components. We confirmed that both AZ1 and AZ2 bind to ACLY and AZ colocalizes with ACLY to the cytoplasm. Unexpectedly, neither AZ1 nor AZ2 accelerated ACLY degradation. Additionally, purified AZ, particularly AZ1, increased the activity of purified ACLY in a dose-dependent manner *in vitro*, suggesting that AZ activates ACLY through protein–protein interaction. Polyamines themselves had no effect on the ACLY activity *in vitro*. Knockdown of AZ1 and/or AZ2 in human cancer cells significantly decreased the ACLY activity as well as cellular levels of acetyl-CoA and cholesterol. Our results are the first to show the crosstalk between polyamine and acetyl-CoA metabolism. We hypothesize that AZ may promote acetyl-CoA synthesis to downregulate spermidine and spermine through acetylation.

1. Introduction

Polyamines (i.e., putrescine, spermidine, and spermine) are essential for cell proliferation and regulation of various cellular processes [1]. High concentrations of cellular polyamines have been reported in various types of cancer [2]. Antizyme (AZ) is a negative regulator of cellular polyamines. AZ is induced by polyamines through a unique mechanism, translational frameshifting [3]. AZ binds to ornithine decarboxylase (ODC), a key enzyme in polyamine synthesis, and accelerates the degradation of the enzyme protein by the 26S proteasome [4]. AZ also inhibits the cellular uptake of polyamines [5]. By blocking both the biosynthesis and uptake, AZ quickly and strongly suppresses the cellular polyamine supply. AZ has also been shown to promote polyamine excretion [6]. The overexpression of AZ has been shown to lead to growth inhibition of cells and exerts an anti-tumor activity [7]. Three types of AZ exist in mammals: AZ1 and AZ2 are expressed in most tissues, whereas AZ3 is observed only in the testis [8]. The distribution of AZ1 and AZ2 overlaps, and the expression level of AZ1 is much higher than that of AZ2 [9]. Recent studies have reported that AZ1 also binds to and accelerates the degradation of other functional proteins such as Smad1, cyclin D1 and Aurora-A [10–12]. Another AZ-binding protein is antizyme inhibitor (AZI), an ODC-related protein, which binds to AZ in competition with ODC [13]. The binding of AZ stabilizes AZI [14].

ATP citrate lyase (ACLY) is a cytosolic enzyme that catalyzes the generation of acetyl-CoA from citrate [15]. Acetyl-CoA is the building block for the biosynthesis of fatty acids and cholesterol, and also serves as a substrate of the acetylation reaction of various molecules, including histones and polyamines [16–18]. Lipid anabolism and histone acetylation are generally associated with cell proliferation, and indeed ACLY is activated in various types of cancer [18]. The inhibition of ACLY induces proliferation arrest in cancer cells [19,20]. Although ACLY is mainly regulated at the

Abbreviations: AZ, antizyme; ODC, ornithine decarboxylase; ACLY, ATP citrate lyase.

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transcriptional level [21], it is also known that ACLY is degraded by the 26S proteasome and ubiquitination and acetylation of the same lysine residue determines the stability of ACLY [22]. In addition to these quantitative regulation mechanisms, ACLY is activated by phosphorylation at Ser-455, which is catalyzed by PI3K/Akt pathway [19].

During screening of AZ-interacting proteins, we found that ACLY bound to AZ. ACLY is regulated by AZ in a manner completely different from any other AZ-binding proteins. The novel crosstalk between polyamine and acetyl-CoA metabolism may have physiological significance in polyamine regulation and cancer biology.

2. Material and methods

2.1. Cell lines

FreeStyle™ 293-F cells (Thermo Fisher Scientific) were cultured in FreeStyle™ 293 Expression Medium (Thermo Fisher Scientific). Human lung cancer-derived A549 cells were grown in Dulbecco's modified Eagle's medium (Wako, Japan) supplemented with 10% fetal bovine serum (Gibco) and non-essential amino acids (Gibco). Human prostate carcinoma-derived LNCaP cells were cultured in RPMI 1640 medium (Wako) supplemented with 10% fetal bovine serum. All cells were cultured at 37 °C in 5% CO_2.

2.2. Construction of plasmids

Human AZ1, AZ2, ACLY, and ODC cDNAs were amplified by PCR from Gene Pool™ cDNA of human normal brain tissue (Thermo Fisher Scientific). The T base at the frameshift site of AZ cDNA was deleted for the frameshift-independent expression [3]. To generate FLAG-tagged constructs, fragment of AZ1 or AZ2 cDNA was inserted into the p3xFLAGCMV-7.1 Vector (Sigma–Aldrich). For HA-tagged constructs, AZ1, AZ2, ACLY or ODC fragment was inserted into the corresponding sites of the pCMV-HA Vector (Clontech).

2.3. Transfection and RNA interference

Transfection of 293-F cells with HA- or FLAG-tagged cDNAs was performed using 293fectin™ (Thermo Fisher Scientific) according to the manufacturer's instructions. The small interfering RNA (siRNA) oligonucleotide for ACLY (M004915) was purchased from GE Healthcare, and those for AZ1 (Hs-OAZ1-6270) and AZ2 (Hs-OAZ2-6643) from Sigma–Aldrich. All of these siRNAs are designed to minimize the off-target effects. Randomized negative control siRNA (Cosmo Bio, Japan) was used as a negative control in all the RNA interference experiments. Transfection of siRNA was performed using 10 nM oligonucleotides and Lipofectamine™ RNAiMAX transfection reagent (Thermo Fisher Scientific) according to the manufacturer's instructions.

2.4. Immunoprecipitation assay and western blotting

The 293-F cells were transfected with indicated plasmids. The cells were harvested at 24 h post-transfection and centrifuged at 300 × *g* for 3 min at 4 °C. The cells were then washed in cold PBS twice and scraped in RIPA buffer (20 mM Tris–HCl, pH7.6, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.1% sodium deoxycholate, 0.1% SDS, and 50 mM sodium fluoride) supplemented with a protease inhibitor cocktail (Roche). The cell lysates were sonicated for 12 s, placed on ice for 15 min, and centrifuged at 12,000 × *g* for 20 min at 4 °C. The protein concentrations of the supernatants were determined by the BCA Protein Assay kit (Thermo Fisher Scientific). Cellular proteins were immunoprecipitated by anti-FLAG M2 affinity gel (Sigma Aldrich), separated on a 12.5% polyacrylamide-SDS gel (ATTO, Japan), and transferred to a PVDF membrane. The blots were probed with the indicated antibodies, and the protein signals were detected by ImmunoStar LD (Wako) and analyzed on the LAS-4000mini system (Fujifilm, Japan) with the Multi Gage V3.0 software program. The primary antibody used was polyclonal anti-HA antibody (1:3,000, Cell Signaling Technology) or polyclonal anti-FLAG antibody (1:20,000, Cell Signaling Technology). The secondary antibody was peroxidase-conjugated anti-rabbit IgG (1:10,000; GE Healthcare). These experiments were repeated at least three times and representative data are presented.

2.5. Real-time RT-PCR

Total RNA was extracted from A549 or LNCaP cells using Total RNA Isolation kit (Thermo Fisher Scientific) and cDNA was synthesized using Superscript VILO cDNA synthesis kit (Thermo Fisher Scientific) according to the manufacturer's protocol. Real-time RT-PCR was performed with TaqMan Master Mixes (Thermo Fisher Scientific) and TaqMan Gene Expression Assays (Thermo Fisher Scientific); AZ1 (Hs00427923_m1), AZ2 (00159726_m1), ACLY (Hs00982738_m1), and GAPDH Control Reagents (402869) using a StepOnePlus real-time PCR system (Applied Biosystems).

2.6. Immunofluorescence

The transfected cells were plated on fibronectin-coated glass-bottom dishes, and 1 h later fixed with methanol and permeabilized in acetone. FLAG-tagged AZ1 or AZ2 and HA-tagged ACLY were detected with primary anti-FLAG mouse antibody (1:1000, Sigma–Aldrich) and primary anti-ACLY rabbit antibody (1:1000, Abcam), followed by Alexa Fluor 488 goat anti-mouse (1:100, Life Technologies) and Alexa Fluor 594 donkey anti-rabbit (1:100, Life Technologies) secondary antibodies, respectively. Nuclei were stained using media containing Hoechst 33342 (Thermo Fisher Scientific). Images were acquired on a BZ-8100 fluorescence microscope (Keyence) at 60× magnification with an oil lens.

2.7. Degradation assay

The 293-F cells were transfected with indicated plasmids and cycloheximide (CHX, 50 μg/ml) was added to the medium at 24 h post-transfection. Cell extracts were prepared as described in 2.4 and subjected to a Western blot analysis.

2.8. ACLY activity assay

The ACLY activity was measured using the malate dehydrogenase coupled method as described previously [19,23]. The cells were washed twice in cold PBS and scraped in RIPA buffer. The lysates were placed on ice for 15 min and then centrifuged at 12,000 × *g* for 20 min at 4 °C. Reaction mixtures containing the appropriate amounts of lysates or purified recombinant proteins were monitored for absorbance at 340 nm while incubating at room temperature using Infinite M200 PRO (TECAN). Changes in absorbance in the absence of exogenous ATP were subtracted from changes in the presence of ATP and normalized to the protein concentration to determine the specific ACLY activity.

2.9. Purification of recombinant proteins

The 293-F cells transfected with either pCMV-HA-ACLY, pCMV-HA-AZ1 or pCMV-HA-AZ2 were harvested at 24 h post-transfection. Recombinant ACLY, AZ1 and AZ2 proteins were purified using the HA tagged protein purification kit (MBL, Japan)

according to the manufacturer's instructions. The protein concentration was measured by the BCA Protein Assay kit.

2.10. Measurement of acetyl-CoA

The cells were harvested by trypsin and washed twice in cold PBS. An internal standard, 2.5 nmol *n*-propionyl-CoA (Sigma-Aldrich), was added to the harvested cells and 10% ice-cold trichloroacetate (TCA) was added to the samples. Following vigorous vortex mixing for 3 min, the TCA suspension was centrifuged at 10,000 × *g* for 10 min at 4 °C. The supernatants were extracted with ice-cold diethyl ether to remove TCA. The dried extract was dissolved in 50% methanol and 0.01% ammonium formate. Ten μL of samples were injected into a liquid chromatography/tandem mass spectrometer (LC-MS/MS) (BRUKER). Chromatography was conducted with an ACQUITY UPLC BEH C18 column (particle size 1.7 μm, 2.1 × 50 mm, Waters). The mobile phases of chromatography were 90% 10 mM ammonium formate, 10% methanol (solvent A) and 10% 10 mM ammonium formate, 90% methanol (solvent B). The mass spectrometer was operated in the high-resolution positive ion mode and isolated molecular ions and fragment ions were analyzed with the BRUKER data analysis software program. The data were normalized to the cell count and the internal standard.

2.11. Measurement of cholesterol

Cholesterol was extracted according to the method described previously with slight modification [24]. One nmol of Cholesterol-2,2,3,4,4,6-d_6 (CDN Isotopes, Canada) was added as an internal standard. The dried extract dissolved in 100 μl of chloroform/methanol (2:1) and 2 μl were injected into the LC–MS/MS. Chromatography was conducted with a Raptor™ LC Biphenyl Column (particle size 2.7 μm, 150 × 2.1 mm, Restek). The LC mobile phase was acetonitrile-methanol (1:4). The fragment ions were detected by QTrap (AB Siex) and cholesterol was measured using the optimized MRM mode. The data were normalized to the cell count and the internal standard.

2.12. Statistical analysis

Experimental data are expressed as the mean ± S.D. The statistical significance of the differences was assessed by Student's *t*-test. A value of $P < 0.05$ was considered to be significant.

3. Results

3.1. AZ binds to and colocalizes with ACLY

ACLY was initially identified in our laboratory as an AZ-interacting protein using yeast two-hybrid screening with AZ2 as the bait (data not shown). We performed an immunoprecipitation assay using FLAG-tagged AZ1, FLAG-tagged AZ2, and HA-tagged ACLY expressed in 293-F cells. As expected, we found that both AZ1 and AZ2 bind to ACLY (Fig. 1*A*).

Next, the subcellular distribution of tagged AZ and ACLY was examined in 293-F cells by cell-imaging techniques using fluorescent microscopy. ACLY and AZ1 were mainly localized to the cytosol, whereas AZ2 was observed in both the cytosol and nucleus. In the cytosol, the localization of AZ and ACLY coincided with one another (Fig. 1*B*). These results are consistent with previous evidence that AZ1 and AZ2 bind to ACLY in the cells.

3.2. AZ does not accelerate the degradation of ACLY

We next examined the effects of AZ on the stability of ACLY in 293-F cells in which *de novo* protein synthesis was inhibited by cycloheximide. The degradation of ODC was accelerated by AZ1 and AZ2, as reported previously [4,25] (Fig. 2, top panel). Under the same condition, the stability of ACLY was not affected by AZ1 or AZ2 (Fig. 2, bottom panel).

3.3. AZ promotes ACLY activity

To determine whether AZ affects the activity of ACLY, HA-tagged ACLY, AZ1, and AZ2 were purified from the cells and the ACLY activity was measured *in vitro* with and without AZ. As shown in Fig. 3*A*, both AZ1 and AZ2 activated ACLY in a dose-dependent manner although AZ1 was much more effective than AZ2. The difference in the efficiency is due likely to the difference in the binding affinity. Next, the effect of AZ on the ACLY activity was tested in cultured cells. We chose two human cancer cell lines, A549 and LNCaP, as it has been reported that the proliferation of these cell lines was markedly suppressed by ACLY knockdown [19,26]. Putrescine (5 mM) was added to the culture media to induce endogenous AZ, and the ACLY activity in the cell extracts was measured after 2 h. As shown in Fig. 3*B*, the ACLY activity was significantly increased upon putrescine in both cells. Addition of spermidine (1 mM) or spermine (1 mM) also increased the cellular ACLY activity (data not shown). Knockdown of either AZ1 or AZ2 siRNA for 48 h reduced the mRNA levels of both AZ1 and AZ2 to 10% and 20% in A549 and LNCaP cells, respectively (Supplementary Fig. 1). Under this condition, siRNA treatment inhibited the effect of putrescine although the effect was weaker for AZ2 than AZ1 (Fig. 3*B*). Double knockdown of AZ1 and AZ2 also inhibited the effect of putrescine to a similar extent. Additionally, the polyamines had no direct effect on the ACLY activity *in vitro* cell free system (Fig. 3*C*). These findings suggest that the activation of ACLY by putrescine was due to the induction of endogenous AZ in cells.

3.4. Knockdown of AZ downregulates ACLY activity and reduces cellular acetyl-CoA and cholesterol levels

We then tested the effect of knockdown of ACLY or AZ on the ACLY activity in polyamine-untreated cells. The addition of siRNA against ACLY resulted in 90% and 80% decrease in the ACLY activity in A549 and LNCaP cells, respectively (Fig. 4*A*). Knockdown of AZ1 significantly suppressed the ACLY activity by 40% and 38% in A549 and LNCaP cells, respectively. The effect of AZ2 knockdown was much weaker, suppressing the ACLY activity in A549 and LNCaP cells, respectively, but it was still significant (Fig. 4*A*).

ACLY catalyzes the production of acetyl-CoA from citrate in the cytoplasm, and acetyl-CoA serves as a substrate of cholesterol synthesis. We next investigated whether knockdown of endogenous AZ affects the acetyl-CoA and cholesterol levels in A549 and LNCaP cells. The cellular acetyl-CoA level was decreased by knockdown of either AZ1 or AZ2, similar to knockdown of ACLY, in both cells (Fig. 4*B*). The cellular cholesterol level was also decreased by knockdown of either AZ1 or AZ2 in A549 cells and by knockdown of AZ1 in LNCaP cells (Fig. 4*C*). These results suggest that endogenous AZ affects the biosynthesis of acetyl-CoA and cholesterol through the regulation of ACLY.

4. Discussion

Polyamines are essential for cell proliferation and their concentrations are generally high in cancer cells. ACLY is also related to cell proliferation and is activated in many types of cancer [20]. However, the relationship between polyamine metabolism and ACLY has remained unaddressed. In this study, we demonstrated that AZ, a polyamine-induced negative regulator of polyamines,

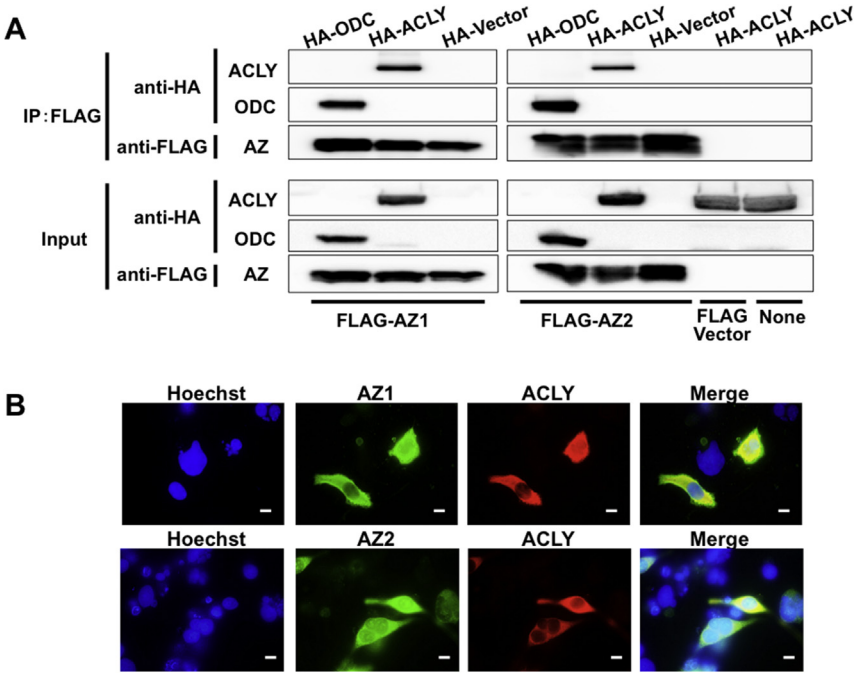

Fig. 1. AZ binds to ACLY. (A) 293-F cells were co-transfected with FLAG-tagged AZ1 or FLAG-tagged AZ2 together with either HA-tagged ODC, HA-tagged ACLY or pCMV-HA (HA-vector). As negative controls, the cells were co-transfected with FLAG-vector without any insert and HA-tagged ACLY, or transfected with HA-tagged ACLY alone. The cells were harvested at 24 h post-transfection. The cell extracts were immunoprecipitated with anti-FLAG M2 affinity gel and subjected to a Western blot analysis using anti-HA antibody (for ODC and ACLY) or anti-FLAG antibody (for AZ) (top panels). Input controls are also shown (bottom panels). The experiments were repeated three times with good reproducibility. (B) 293-F cells co-transfected with FLAG-tagged AZ1 or AZ2 together with HA-tagged ACLY were plated on glass-bottom dishes and observed using an immunofluorescence method. AZ1 or AZ2 was labeled with Alexa Fluor 488 (green), and ACLY was labeled with Alexa Fluor 594 (red). Nuclei were stained with Hoechst 33342 (blue). The images represent the typical localization pattern of AZ and ACLY. Scale bar, 10 μm.

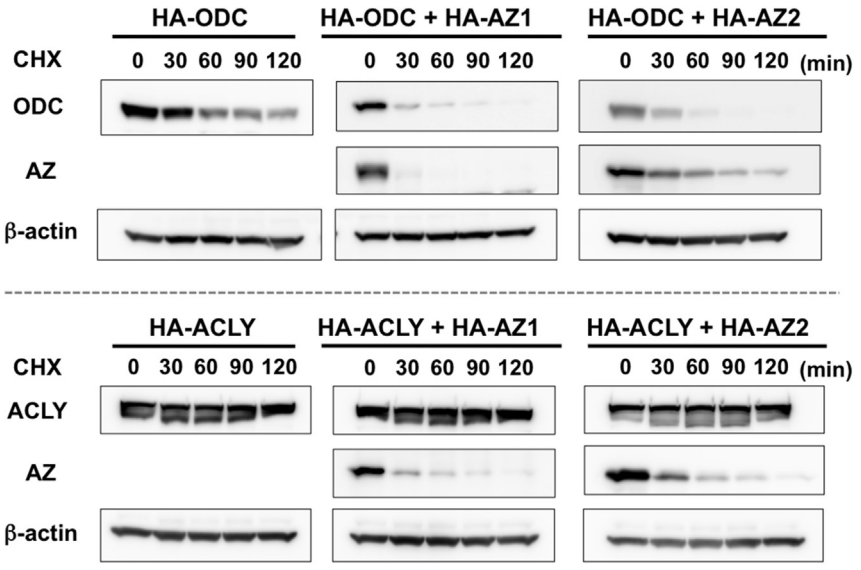

Fig. 2. AZ does not accelerate the degradation of ACLY. 293-F cells were transfected for 24 h with HA-tagged ODC (top panels) or HA-tagged ACLY (bottom panels) together with or without HA-tagged AZ1 or HA-tagged AZ2. Cycloheximide (CHX) was added to the media, the cells were harvested for the indicated times and the cell extracts were subjected to a Western blot analysis using anti-HA antibody. β-actin was detected as a loading control. The experiments were repeated three times with good reproducibility.

binds to ACLY. ACLY was co-immunoprecipitated by both AZ1 and AZ2, and colocalized to the cytoplasm with both AZ1 and AZ2 (Fig. 1*A*, *B*).

It is known that AZ binds to ODC to accelerate the degradation of ODC protein by the 26S proteasome without ubiquitination. It has also been reported that other AZ1-binding proteins such as Smad1, cyclin D1 and Aurora-A are destabilized by AZ1 [10–12]. We had hypothesized that binding of AZ would accelerate the degradation of ACLY; however, the stability of ACLY was hardly affected by AZ-binding (Fig. 2).

We then demonstrated *in vitro* that both AZ1 and AZ2 enhance ACLY activity in a dose-dependent manner (Fig. 3*A*). In the cancer cells, putrescine, an inducer of endogenous AZ, increased the activity of ACLY (Fig. 3*B*). The increase in ACLY activity by putrescine

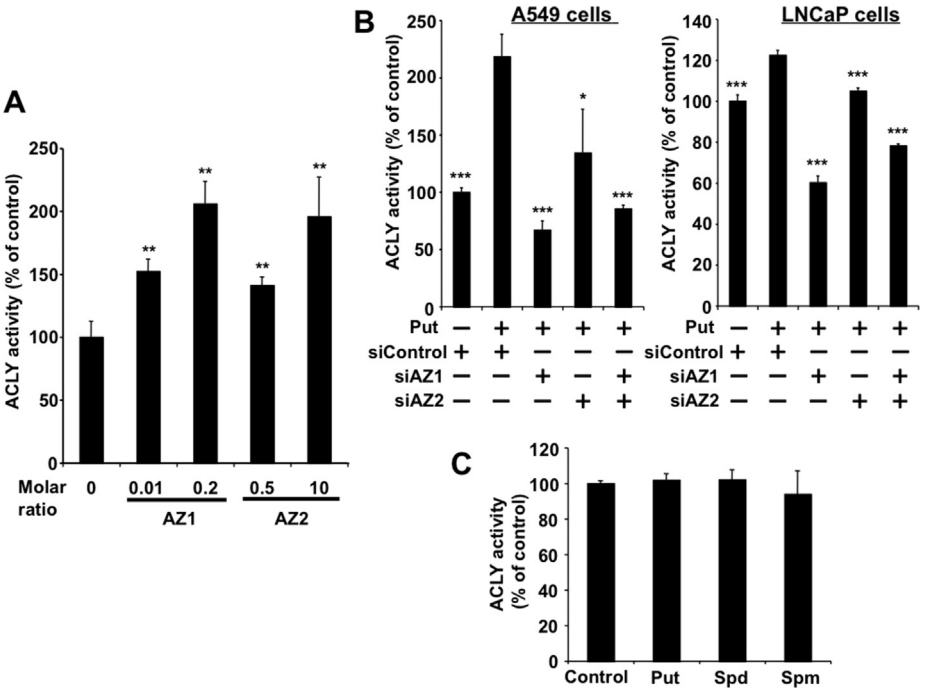

Fig. 3. AZ promotes ACLY activity. (A) The activity of purified ACLY was measured using the malate dehydrogenase coupled method in the presence or absence of purified AZ1 or AZ2. (B) A549 or LNCaP cells were treated with siRNA for AZ1 (siAZ1) and/or AZ2 (siAZ2) or randomized negative control siRNA for 48 h before harvesting. Putrescine (Put) was added to the culture at 5 mM for the last 2 h. Harvested cell extracts were used to measure the ACLY activity using the malate dehydrogenase coupled method. Significance was indicated versus the putrescine control. (C) The activity of purified ACLY was measured *in vitro* in the presence of 5 mM putrescine (Put), 1 mM spermidine (Spd) or 1 mM spermine (Spm). Data are expressed as the mean ± S.D. of 4 experiments. *P < 0.05, **P < 0.01, ***P < 0.001 versus the corresponding control.

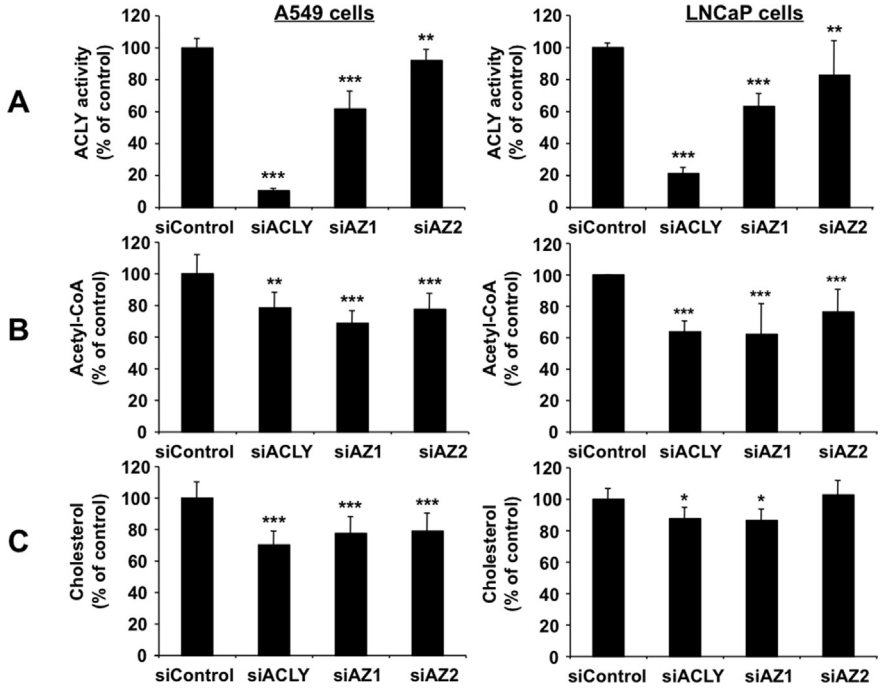

Fig. 4. Endogenous AZ regulates ACLY and its downstream metabolites in cells. (A) Cells were treated by siRNA of ACLY (siACLY), AZ1 (siAZ1), AZ2 (siAZ2), or control siRNA for 48 h before harvesting cells for the ACLY activity assay. (B) Cells were treated as in A. The level of acetyl-CoA is represented as % of the control. (C) Cells were treated as in A. The level of cholesterol is represented as % of the control. Left panels represent the effect in A549 cells, and right panels in LNCaP cells. Data are expressed as the mean ± S.D. of 4–8 experiments. **P < 0.01, ***P < 0.001 versus the control.

was inhibited by AZ1 knockdown and partially inhibited by AZ2 knockdown, indicating that AZ directly enhances the activity of ACLY, likely through a protein–protein interaction. The molecular mechanism for the activation of ACLY by AZ is of great interest and should be studied in future investigations.

Knockdown of AZ1 or AZ2 in putrescine-untreated cells

significantly decreased the ACLY activity (Fig. 4*A*). Thus, ACLY should be partially activated by endogenous AZ in the cells under physiological conditions. The reduction in ACLY activity was greater following AZ1 knockdown than AZ2 knockdown. This is likely because the expression of AZ1 mRNA in mammalian cells is much higher than that of AZ2 mRNA [9]. Our results also indicated that the intracellular level of AZ1 mRNA was 13-fold and 7-fold higher in LNCaP cells and A549 cells, respectively, than that of AZ2 mRNA (data not shown).

Under the same condition, the knockdown of AZ1 or AZ2 significantly decreased the cellular levels of acetyl-CoA, a product of ACLY (Fig. 4*B*). In A549 cells, AZ knockdown also lowered the cellular level of cholesterol, which is synthesized from acetyl-CoA, although the decrease was slight in LNCaP cells. The reason for this difference is unclear, however, lipid metabolism might be quantitatively different between the two cancer cell lines.

Polyamines are basic molecules, and spermidine and spermine in particular bind to cellular acidic molecules, such as nucleic acid. In proliferating cells, such acidic molecules increase and more polyamines are required. Meanwhile, excessive concentrations of polyamines are cytotoxic, inducing cell death, thus cells are equipped with feedback regulatory mechanisms for polyamines. AZ suppresses both polyamine synthesis and the uptake of extracellular polyamines. Cellular spermidine and spermine induce spermidine/spermine-N^1-acetyltransferase (SSAT), which acetylates polyamines for further catabolism by acetylpolyamine oxidase or excretion from the cells [17,27]. In SSAT transgenic mice, cytosolic acetyl-CoA is depleted by the acetylation of spermidine and spermine, leading to a decreased synthesis of fatty acid and lean phenotype [28]. Thus, in cells with high spermidine and spermine concentrations, the cytosolic acetyl-CoA requirement must be higher. It is possible that the significance for enhancing the ACLY activity by AZ is to provide acetyl-CoA as the substrate of SSAT. Intracellular concentration of acetyl-CoA has been shown to be relevant to diverse cellular activities including cell proliferation [18].

It has been shown that ACLY is localized to not only the cytoplasm but also the nucleus. Nuclear ACLY may supply acetyl-CoA for histone acetylation, leading to global regulation of the gene expression [16,18]. We previously reported that AZ2 is also localized to both the cytoplasm and nucleus whereas AZ1 is mainly localized to the cytoplasm [25]. Although we did not confirm the co-localization of AZ2 and ACLY to the nucleus in the present study, AZ2 may have a specific role in regulating nuclear ACLY.

In conclusion, our results demonstrated a novel role of AZ with a previously unknown mechanism. Further study would contribute to better understanding of the role of AZ in cancer biology.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2016.02.084.

Conflict of interest statement

The authors declare that they have no conflicts of interest.

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